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(54) **Water-soluble extract of asiaticoside and madecassoside from centella asiatica and isolating method thereof**

Wasserlösliches Extrakt von Asiaticosid und Madecanoside von Centella asiatica und Verfahren zur Isolierung davon

Extrait hydrosoluble de asiaticoside et madécassoside de Centella asiatica et méthode pour leur isolation

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Description**BACKGROUND OF THE INVENTION**5 **Field of the invention**

[0001] The present invention relates to a water-soluble extract of asiaticoside and madecassoside from Centella asiatica, which is able to protect liver cells with an anti-fibrosis effect, and to an isolating method thereof.

10 **Description of the Prior Art**

[0002] Centella asiatica (L.) Urb, a family of umbelliferae which is autogenous in Madagascar island of Africa and India, has been used for the treatment of a local wound since a long time ago (Poizot. A., Dumez. D, C.R. Acad. Sci [D] 286, 1978). Many researchers report that the titrated extract of Centella asiatica (hereinafter referred to as "TECA") is effective to a local injury of the tissues. In fact, TECA is acknowledged as a medicinal effector and commercially available under the tradename of "Madecassol" (asiaticoside : asiatic acid : madecasic acid = 4:3:3). Its medicinal effect is to restore a damaged tissue to a nearly original state by modifying the fibrosis progress with protection of the cells in the tissue.

[0003] These components, however, are limited to the curing of surgical wound and their extraction, as disclosed in 20 Korean Pat. Publication Nos. 87-1458, 87-1573 and 91-2518, requires elaborate processes, so that the yield and purity thereof is poor. In addition, the objective main components are genin (asiatic acid, madecasic acid and madasiatic acid) and asiaticoside, which are both water-insoluble so that there is a limit in expanding their uses.

SUMMARY OF THE INVENTION

[0004] Although the pre-existing TECA exhibits an excellent effect of anti-fibrosis, its physical property of water insolubility forces itself to be in the form of powder, limiting its application range to the curing of surgical wound.

[0005] Intensive research repeated by the present inventors aiming to expand the pharmaceutical use of TECA, resulted in the finding that a treatment of Centella asiatica with an aqueous alcohol greatly helps extract the components which are soluble in water and effective in the protection and the anti-fibrosis of liver cells.

[0006] Therefore, it is an object of the present invention to provide a water-soluble extract of asiaticoside and madecassoside from Centella asiatica, which shows an excellent effect of the protection and the anti-fibrosis of liver cells.

[0007] It is another object of the present invention to provide an isolating method of the water-soluble extract.

[0008] In accordance with an aspect of the present invention, there is provided a method for isolating a water-soluble extract of asiaticoside and madecassoside from Centella asiatica, comprising the steps of: subjecting an aqueous alcohol containing Centella asiatica to cold-precipitation; treating the extract in the aqueous alcohol with a halogenide solvent for layer separation; extracting the aqueous layer with a higher alcohol; washing the extract in the alcohol layer with sodium hydroxide, then with water and concentrating the extract; crystallizing the extract with ethyl acetate, to give a crystal; and washing the crystal.

[0009] In accordance with another aspect of the present invention, there is provided a water-soluble extract of asiaticoside and madecassoside from Centella asiatica, which is able to protect liver cells with an anti-fibrosis effect.

DETAILED DESCRIPTION OF THE INVENTION45 **1. Isolation of the Extract of Asiaticoside and Madecassoside**

[0010] Centella asiatica is immersed in an alcohol solution in water, to give an extract solution which is, then, treated with a halogenide solvent. The aqueous layer was extracted with an alcohol ($C_n \geq 4$). Subsequently, the extract in the alcohol layer was washed several times with an aqueous alkaline solution, and concentrated in vacuo or subjected to precipitation, to give a solid.

[0011] Showing a solubility in water, this solid mainly comprises asiaticoside and madecassoside, represented by the following formula I:

[0012] The alcohol solution used in the present invention comprises a methanol or ethanol with an alcohol content ranging 50-80%. The halogenide solvent useful in the present invention is selected from the group consisting of methylene chloride, chloroform, dichloroethane, and dichloroethene. For the extraction from the aqueous layer, an alcohol containing at least 4 carbon atoms, such as that selected from the group consisting of normal butanol, sec-butanol and amyl alcohol, is used. As the alkaline solution, an aqueous sodium hydroxide or potassium hydroxide solution is employed.

[0013] The extract from *Centella asiatica* obtained by the above procedure consist mainly of asiaticoside and madecassoside with a ratio ranging from 4:6 to 6:4, and it is found that the two components constitute 97% or more of the extract. It can be dissolved in water, alcohol and vegetable oil. Particularly, because the solubility of the extract in water is a very important physical property, it is called water soluble TECA (hereinbefore referred to as "WA-TECA") in the present invention.

2. Test for the Protection of Liver Cells

[0014] After carbon tetrachloride (CCl_4), known as a representative toxic material, and galactosamine were used to induce a toxicity in the primarily cultured liver cells of wistars, WS-TECA was tested for the effect of toxicity interception and restoration.

A) Test animal

[0015] Wistars (male, 150-200 g) were allowed to eat feed and water as much as they like at a temperature of $22\pm 1^{\circ}C$ and at a humidity of $60\pm 5\%$ while day and night are changed every 12 hours. They are starved since a day before the test.

B) Culture of the liver cell of the wistars

[0016] The liver cells were isolated by a two-stage collagenase perfusion technique, a little modified Berry-Friend method. The wistar was put under anesthesia with urethane (1g/kg weight body) and its abdomen was cleaned with 70% ethanol and cut open. The portal fissue vein was intubated with a 20 gauge catheter through which 150 ml of HBBS was allowed to flow at a rate of 15 ml/min. In this while, its inferior vena cava was cut to remove the blood. After its thorax was open and a 18 gauge catheter was inserted into the inferior vena cava, it was corded for the re-circulation of a digestive juice comprising 0.05% collagenase in 95 ml of HBBS. The re-circulation was carried out for 10 min while supplying a mix of CO_2 (5%) and O_2 (95%). When liver cells were separated, the liver was pick out and placed in 60 ml of HBBS in a beaker. The membrane surrounding the liver was ripped open with a pair of scissors to make the liver cells free. Thereafter, they were filtered through a sheet of lens paper. The filtrate thus obtained was centrifuged at 50 g for 2 min. The supernatant was removed out, followed by the centrifugation with a culture medium at the same condition, to give a suspension of liver cells. This suspension was transferred at a concentration of 5×10^5 cells/ml in a collagen-coated culture dish. The culture medium used was a Waymouth's MB 752/1 medium containing 10% fetal calf serum, 2.0 mg/ml bovine serum albumin (fraction V), 10^{-6} M dexamethasone, 10^{-7} M insulin, 5.32×10^{-2} M L-serine, 4.07×10^{-2} M $NaHCO_3$, 100 IU/ml penicillin, 100 μ g/ml streptomycin and 5 μ g/ml amphotericin B. The cells were cultured at $37^{\circ}C$ in an incubator with a constant humidity while supplying a mix of air (95%) and CO_2 (5%).

C) Test Method

[0017] After liver cells were cultured for 24 hours, the culture medium was changed by a fresh medium containing 10 mM carbon tetrachloride and a further culture for 1.5 hours allowed the carbon tetrachloride to induce a toxicity in them. Galactosamine also could induce a toxicity in liver cells by changing a fresh medium containing 1.5 mM galactosamine for a medium in which the liver cells had been cultured for 1.5 hours and culturing them for 14 hours.

[0018] Glutamic pyruvate transaminase (GPT) and sorbitol dehydrogenase (SDH) in the culture media were measured by the Reitman-Frankel method and a little modified Gerlach method.

[0019] The activity of glutathione-S-transferase was represented by the amount of the product produced by 1 mg of the protein per minute, which was measured by mixing a post mitochondrial supernatant in a test tube containing glutathione, 1-chloro-2,4-dinitrobenzene and potassium phosphate buffer and immediately measuring a vertically ascendent absorbance at 340 nm for 5 min.

[0020] The post mitochondrial supernatant was obtained by culturing liver cells, removing the medium, adding 3 ml of a 66 mM Tris-HCl buffer (pH 7.4) to give a suspension of cells, subjecting the suspension to homogenization for 15 sec, and centrifuging the suspension at 12500 g for 15 min.

[0021] A modified McCord-Frividich method was used to measure the activity of superoxide dismutase (SOD) in liver cells. 100 μ l of the pose mitochondrial supernatant was added with 200 μ l of reagent A, 500 μ l of delonized water, and 200 μ l of reagent B, and allowed to stand at $37^{\circ}C$ for 30 min. After the resulting supernatant was again added with 200 μ l of reagent C and allowed to stand at room temperature for 20 min, the absorbance at 550 nm was measured to calculate SOD unit.

[0022] For the activity of GSSG reductase, 200 μ l of the post mitochondrial supernatant was added in a test tube containing 600 μ l of a phosphate buffer, 100 μ l of 10 mM GSSG and 100 μ l of 1 mM NADPH and the change in the

absorbance at 340 nm for 2 min was detected.

[0023] For the measurement of the total content of glutathione (GSH and GSSG), 700 µl of 0.3 mM NADPH and 100 µl of 6 mM DTNB were added to 200 µl of the post mitochondrial supernatant which was, then, incubated at 30 °C in a water bath. After the initiation of reaction by adding 10 µl of GSH reductase (50 units/ml), the change in the absorbance at 412 nm was detected every 15 sec for 2 min.

[0024] As for the content of glutathione (oxidized form, GSSG), a mixture of 2 µl of 2-vinylpridine per 100 µl of the post mitochondrial supernatant was vigorously stirred and allowed to stand at 25 °C for 60 min to produce a GSH derivative whose content was, then, measured for the calculation of the residual GSSG.

[0025] To detect RNA biosynthesis, [³H]-uridine was added to a concentration of 1 µCi/ml of culture medium and the incorporation of the radio-labeled uridine into the RNA of culture cells was measured. For this, after the culture medium was removed, the cells are washed three times with HBSS and added with 1 ml of 10% trichloroacetic acid to precipitate proteins. 1 ml of a mix of ethanol-ether (3:1) was added to remove residual trichloroacetic acid and the proteins were dissolved in 200 µl of 1N NaOH. 100 µl of the protein solution was taken and added to 3 ml of aquasol, a scintillation cocktail for the measurement of radioactivity.

D) Results

[0026] WS-TECA was administered to primarily cultured liver cells of the wistar which had been toxicity-induced by carbon tetrachloride, at an amount ranging from 1 µg/ml up to 300 µg/ml. Showing a dose-dependent effect from 1 µg/ml up to 100 µg/ml, WS-TECA reduced the activity of GPT and SDH released into culture medium with a maximal protection activity of liver cell being showed at 100 µg/ml. In addition, WS-TECA increased the activities of GSSG reductase and SOD, which played a role in hindering the production of the GST free radicals taking part in the detoxification of various enzymes in the liver and in removing the free radicals produced. Whereas, WS-TECA increased the amount of GSH reduced owing to the free radicals. As mentioned above, WS-TECA exhibited a maximal activity of 50-60 % at 100 µg/ml (see Table 1).

[0027] When the primarily cultured liver cells of wistar which had been led to toxicity by galactosamine, was treated with an amount of 1 µg/ml up to 300 µg/ml of WS-TECA, the activities of GPT and SDH were maximally inhibited at 300 µg/ml.

[0028] As for the influence of WS-TECA upon the RNA synthesis in the liver cells damaged by galactosamine, the liver cells were increased in RNA biosynthesis twice upon the addition of 300 µg/ml of WS-TECA as much as upon the addition of no WS-TECA and thus, up to 40 % of the damaged liver cells were restored to the normal state.

TABLE 1

Condition	Protection (%)*					
	GPT	SDH	GST	GSH	GSSG	SOD
Control**	100	100	100	100	100	100
Reference***	0	0	0	0	0	0
WS-TECA 1µg/ml	10.1	3.0	2.8	6.6	13.9	15.8
WS-TECA 10µg/ml	26.9	14.9	5.4	12.7	55.8	31.6
WS-TECA 50µg/ml	35.2	34.3	36.8	26.1	60.0	36.8
WS-TECA 100µg/ml	64.5	55.8	49.2	57.0	63.9	57.9
WS-TECA 300µg/ml	53.6	41.7	38.0	45.5	55.8	47.4

* Protection (%)=(Reference-Sample)/(Reference-Control)

** control : untreated liver cells

*** reference ; CCl₄-treated liver cells

TABLE 2

Condition	Protection (%)*		RNA synthesis (cpm)
	GPT	SDH	
Control**	100	100	615±48
Reference***	0	0	125±11
WS-TECA 1μg/ml	10.8	12.2	109±32
WS-TECA 10μg/ml	18.5	19.1	204±29
WS-TECA 100μg/ml	30.8	32.7	234±14
WS-TECA 300μg/ml	41.5	43.9	267±58

* Protection (%)=(Reference-Sample)/(Reference-Control)

** control : untreated liver cells

*** reference : galactosamine-treated liver cells

E) Anti-Fibrosis Effect

[0029] A test animal was induced to be of liver fibrosis by a bile-duct ligation technique or by the treatment of carbon tetrachloride. WS-TECA obtained in the present invention was administered to a test animal to quantitatively evaluate serum procollagen N-terminal peptide (PIIIP) and hydroxyproline, thus measuring the collagen biosynthesis and accumulation in tissue, a main factor causing the fibrosis process.

[0030] Test procedures and the anti-fibrosis of WS-TECA were as follows.

[0031] The bile duct ligation method was carried out in such a way that the abdomen of a male SD rat (200-220 g) was cut open and the bile duct was subjected to double ligation for 4 weeks to induce the fibrosis of liver cells. A drug was peritoneally administered once a day. Using a rabbit anti-PIIIP antibody, the PIIIP in serum was quantitatively measured by an ELISA method. The quantitation of the collagen in the liver tissue was performed by a coloring method in which the hydroxyproline released when the liver tissue was acid-hydrolyzed was treated with chloramine T and ER solution (p-dimethylaminobenzaldehyde, perchloric acid, isopropanol).

[0032] As for the anti-fibrosis effect in the bile duct ligation test, WS-TECA could decrease the hydroxyproline amount, an index of liver fibrosis (see Table 3). In addition, the PIIIP concentration in serum, used as an index of collagen biosynthesis, could be inhibited by WS-TECA (see Table 4). As apparent from these facts, WS-TECA was effective for relieving the fibrosis of liver.

[0033] Following is a test of the liver fibrosis induced by carbon tetrachloride.

[0034] CCl₄ was peritoneally administered to a male SD rat at a dose of 480 mg per weight twice a week for 4 weeks. The test drug dissolved in a physiological saline was also peritoneally administered once a day for 4 weeks. The PIIIP in serum and the collagen in liver tissue were quantitated in the same manner with that of the bile duct ligation test.

[0035] The CCl₄ treatment resulted in a more relieved fibrosis induction than does the bile duct ligation and the liver tissue treated with CCl₄ made collagen deposited in itself twice as much as a control, which is treated with no drugs.

[0036] Showing a dose-dependent effect, WS-TECA inhibited the deposition of collagen in liver tissue (see Table 5) as well as lowered the concentration of PIIIP in serum (see Table 6).

[0037] These data demonstrated that WS-TECA might be useful to relieve the liver fibrosis, attributed to the increase of collagen synthesis and the deposition of collagen in liver tissue, by hindering the synthesis of collagen.

TABLE 3

Inhibitory Effect of WS-TECA on Deposition of Hydroxyproline in Liver Tissue	
Group	Total hydroxyproline in Liver Tissue
Control	32.11±6.07
Bile duct-ligated	162.2±127
WS-TECA administered	
1.5 mg/kg	111.2±60.6
5.0 mg/kg	96.4±51.7

TABLE 4

Lowering Effect of WS-TECA on Concentration of PIIIP in Serum	
Group	PIIIP Conc. in Serum
Control	6.1 ± 2.9
Bile duct-ligated	37.4 ± 12.9
WS-TECA administered 1.5 mg/kg	25.5 ± 6.4

TABLE 5

Inhibitory Effect of WS-TECA on Deposition of Collagen in CCl ₄ -treated Rat	
Group	Total hydroxyproline in Liver Tissue
Control	1.67±0.26
CCl ₄ -treated	3.02±0.78
WS-TECA administered 0.5 mg/kg	3.29±1.34
1.5 mg/kg	2.39±0.89
5.0 mg/kg	2.18±1.03

TABLE 6

Lowering Effect of WS-TECA on PIIIP Concentration in Serum in CCl ₄ -treated Rat	
Group	PIIIP conc. in serum
Control	7.4 ± 2.9
Bile duct-ligated	47.2 ± 7.8
WS-TECA administered 0.5 mg/kg	32.8 ± 9.2
1.5 mg/kg	26.1 ± 3.5
5.0 mg/kg	27.6 ± 6.3

[0038] A better understanding of the present invention may be obtained through the following examples which are set forth to illustrate, but are not to be construed as the limit of the present invention.

EXAMPLE I

[0039] To 4.7 kg of dried Centella asiatica was added 32 liters of 70% ethanol which was, then, subjected to cold-precipitation for 48 hours. The resulting extract in ethanol was placed in a bottle, added with 17 liters of methylene chloride and stirred for 1 hour. After complete phase separation, the upper layer was taken, added with 5 liters of ethanol and 7 liters of methylene chloride, and stirred for 1 hour. Again, after complete phase separation, the upper layer was taken and extracted by use of 17 liters of normal butanol. The extract in the normal butanol layer was washed several times with a 0.1 N NaOH solution and then, several times with water. The butanol layer was concentrated in vacuo to one tenth in volume and the resulting concentrate was mixed with 5 liters of ethyl acetate for crystallization. The crystal thus obtained was filtered with suction to obtain a solid residue. Subsequently, it was washed with a little amount of ethyl acetate, dried in vacuo at 50 °C for 24 hours, to yield 120 g of a yellowish solid.

EXAMPLE II

[0040] To 5 kg of Centella asiatica was added 35 liters of 70% ethanol which was, then, allowed to stand at 10 °C for 48 hours.

[0041] The extract in ethanol thus obtained was placed in a bottle and treated in the same manner with that of Example I, to yield 125 g of a mix of asiaticoside and madecassoside (4:6).

EXAMPLE III

[0042] A mix extract of asiaticoside and madecassoside (6:4) was obtained in the same manner with that of Example I.

5 EXAMPLE IV

[0043] A silica gel (70-230 mesh) was filled in a column with a diameter of 6 cm and a length of 26 cm by using a mobile phase (normal butanol:ethanol:ammonia water:water=60:40:5:10). Thereafter, 1 g of WS-TECA was dissolved in as little amount of the mobile phase as possible and loaded thereon. The mobile phase was allowed to flow at a constant rate and fractionated in 25 ml bottles to obtain 400 mg of pure asiaticoside and madecassoside each.

TEST EXAMPLE

[0044] 15 5 g of WS-TECA was dissolved in 100 ml of methanol which was, then, added with 3 ml of 5N NaOH and heated for 12 hours with reflux. The resulting solution and a conventional TECA were developed on a stationary phase with a mobile phase (normal butanol:ethanol:ammonia water=60:40:5:10) and colored by a mix of anhydrous acetic acid and sulfuric acid (9:1), to identify the hydrolysates of WS-TECA with madecassic acid and asiatic acid.

[0045] 20 The HPLC analysis of the hydrolysates showed that asiaticoside and madecassoside were present at a ratio of 5:5.

[0046] 25 In many countries, chlonic liver diseases, such as viral hepatitis and alcoholic hepatitis, occur very frequently every year, recording a high death rate. Such liver diseases continuously bring injury into the liver cells which are, thus, led to liver fibrosis. Up to now, colchichine and UDCA have been tried for treating the liver diseases but there are many different views concerning their effect. Therefore, it is pressing to develop a novel therapeutic agent capable of reducing the damage and fibrosis of liver cells. As described above, the mix of asiaticoside and madecassoside, extracted from Centella asiatica, is expected to be able to reduce the serosity of the chlonic liver diseases as well as the death rate owing to the diseases.

[0047] 30 The present invention has been described in an illustrative manner, and it is to be understood the terminology used is intended to be in the nature of description rather than of limitation.

[0048] 35 Many modifications and variations of the present invention are possible in light of the above teachings. Therefore, it is to be understood that within the scope of the appended claims, the invention may be practiced otherwise than as specifically described.

Claims

- 35 1. A method for isolating a water-soluble extract of asiaticoside and madecassoside from Centella asiatica, comprising the steps of:
 - 40 subjecting an aqueous alcohol containing Centella asiatica to cold-precipitation;
 - treating the extract in the aqueous alcohol with a halogenide solvent for layer separation;
 - extracting the aqueous layer with a higher alcohol;
 - washing the extract in the alcohol layer with sodium hydroxide, then with water and concentrating the extract;
 - crystallizing the extract with ethyl acetate, to give a crystal; and
 - washing the crystal.
- 45 2. A method in accordance with claim 1, wherein said aqueous alcohol is an ethanol or methanol solution, said halogenide solvent is selected from the group consisting of methylene chloride, chloroform and dichloroethane, and said higher alcohol is selected from the group consisting of normal butanol, sec-butanol and amyl alcohol.
- 50 3. A water-soluble extract of asiaticoside and madecassoside from Centella asiatica, which is able to protect liver cells with an anti-fibrosis effect.
4. A water-soluble extract in accordance with claim 3, wherein said asiaticoside and said madecassoside are in a ratio ranging from 4:6 to 6:4.

Patentansprüche

1. Ein Verfahren zum Isolieren eines in Wasser löslichen Extraks von Asiaticosid und Madecassosid aus Centella asiatica, umfassend die Schritte:

5 Unterwerfen eines Centella asiatica enthaltenden wässrigen Alkohols der Kaltausfällung;

10 Behandeln des Extraks in dem wässrigen Alkohol mit einem Halogen-Lösungsmittel zur Schichttrennung;

15 Extrahieren der wässrigen Schicht mit einem höheren Alkohol;

Waschen des Extraks in der Alkoholschicht mit Natriumhydroxid, dann mit Wasser und Konzentrieren des Extraks;

20 Kristallisieren des Extraks mit Ethylacetat zur Kristallbildung; und

Waschen der Kristalle.

25 2. Ein Verfahren gemäß Anspruch 1, worin der wässrige Alkohol eine Ethanol- oder Methanol-Lösung ist, das Halogen-Lösungsmittel ausgewählt ist aus der Gruppe bestehend aus Methylchlorid, Chloroform und Dichlorethan, und der höhere Alkohol ausgewählt ist aus der Gruppe bestehend aus n-Butanol, sec-Butanol und Amylalkohol.

30 3. Ein wasserlöslicher Extrakt von Asiaticosid und Madecassosid aus Centella asiatica, welcher befähigt ist, Leberzellen durch eine Antifibrosewirkung zu schützen.

35 4. Ein wasserlöslicher Extrakt gemäß Anspruch 3, worin das Asiaticosid und das Madecassosid in einem Verhältnis im Bereich von 4 : 6 bis 6 : 4 vorliegen.

Revendications

30 1. Procédé d'isolement d'un extrait hydrosoluble d'asiaticoside et de madécassoside de Centella asiatica, comprenant les étapes consistant à :

35 soumettre un alcool aqueux contenant Centella asiatica à une précipitation à froid;
traiter l'extrait dans l'alcool aqueux avec un solvant halogénure pour une séparation de phase;
extraire la phase aqueuse avec un alcool supérieur;
laver l'extrait de la phase alcoolique avec de l'hydroxyde de sodium, puis avec de l'eau et concentrer l'extrait;
cristalliser l'extrait avec de l'acétate d'éthyle, pour donner un cristal; et
laver le cristal.

40 2. Procédé selon la revendication 1, dans lequel ledit alcool aqueux est une solution d'éthanol ou de méthanol, ledit solvant halogénure est choisi dans le groupe comprenant le chlorure de méthylène, le chloroforme et le dichloroéthane, et ledit alcool supérieur est choisi dans le groupe comprenant le butanol normal, le sec-butanol et l'alcool amylique.

45 3. Extrait hydrosoluble d'asiaticoside et de madécassoside de Centella asiatica, qui est capable de protéger les cellules du foie par un effet anti-fibrose.

50 4. Extrait hydrosoluble selon la revendication 3, dans laquelle ledit asiaticoside et ledit madécassoside sont dans un rapport allant de 4:6 à 6:4.